



# Affordable and sensitive determination of artemisinin in *Artemisia annua* L. by gas chromatography with electron-capture detection

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## ABSTRACT

Artemisinin demand has increased sharply since the World Health Organization recommended its use as part of the artemisinin combination therapies in 2001. The area for the crop cultivation has expanded in Africa and Asia and simpler and affordable methods for artemisinin analysis are needed for crop quality control. This work presented a novel chromatographic method of artemisinin analysis using gas chromatography with electron-capture detection. The sample extraction and preparation involved a single-solvent one-step extraction, with samples being analyzed in the extraction solvent directly after extraction. This method was accurate and reproducible with over 97% recoveries. The limit of detection was less than 3 µg/mL and the limit of quantification was less than 9 µg/mL, allowing samples as low as 100 mg dry weight to be analyzed for artemisinin. The method can be applied to quality control of commercial plant extracts and to artemisinin-derived pharmaceuticals.

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## 1. Introduction

Artemisinin is an endoperoxide-containing sesquiterpene lactone isolated from the aerial parts of *Artemisia annua* L. (sweet wormwood), a herb of the Asteraceae family that has been used for centuries in China for the treatment of fever and chills associated with malaria [1]. It is currently the best therapy against drug-resistant strains of *Plasmodium falciparum*, which cause cerebral malaria and death [2]. Malaria is one of the most important parasitic diseases in the world, affecting at least 300 millions people a year, and resulting in more than a million deaths [3,4]. The multi-drug-resistant *Plasmodium* strains, mutated against the most affordable antimalarials currently used, such as chloroquine, mefloquine and sulfadoxine-pyrimethamine, and represent one of the biggest challenges in fighting malaria in developing nations [3]. Due to its high efficacy, fast action, low-toxicity, and no serious side effects or adverse reactions, artemisinin is regarded as 'a breakthrough in the history of antimalarial drugs' [5].

Besides malaria, artemisinin proved effective against hepatitis B [6], schistosomiasis [7], several blood parasitic protozoans [8–10], and against a variety of cancer cell lines including breast cancer, human leukemia, colon, small-cell lung carcinomas [11] and drug-resistant cancers [12].

Various methods have been developed to quantify artemisinin. Thin layer chromatography is not a reliable technique to quantify artemisinin due to the poor staining characteristics of the intact molecule and interference of other constituents in the plant matrix [13,14]. Due to the absence of appropriate UV absorbance, a pre-[15] or post-column [16] derivatization procedure is required for high performance liquid chromatography with UV detection (HPLC-UV) or diode array detection (HPLC-DAD) [17], which increases sample preparation labor, analysis cost, and other compounds similar to artemisinin (such as artemisitene) might also get derivatized leading to over-estimation and lack of specificity. Direct analysis of artemisinin without sample derivatization by HPLC with evaporative light scattering detection (ELSD) has been developed by several research groups [18–20], but its sensitivity to artemisinin is low when compared to HPLC-ED (electrochemical detection) and HPLC-MS. Quantification by HPLC-ED [21–23] is sensitive and specific, but oxygen must be removed from the mobile phase by continuously purging with helium or argon. Even when oxygen has been eliminated, it takes over 1 h to stabilize the baseline before any injection can be made. Several HPLC-MS [24–28] and HPLC-MS/MS [29,30] have been developed to analyze (dihydro) artemisinin in blood, plasma, serum or *A. annua*. But both the MS and MS/MS detectors are expensive, costly to maintain, and they require great expertise. GC usually uses nitrogen or helium as mobile phase, eliminating harmful organic solvent disposal, whereby it offers an economic and environmentally friendly determination method. Although thermally unstable compounds, such as (dihydro) artemisinin, cannot be directly determined by GC, they

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can be quantified through derivatization or their stable degradation products. GC-flame ionization detection (FID) [19,22,31] and GC-MS [32–35] analyses of (dihydro) artemisinin, indirectly by detecting one [19,31,32] or all [22,33,34] of its major decomposition products, have been established. However, the sensitivity of GC-FID for artemisinin determination is limited and the cost of GC-MS is high.

Most sample preparation techniques currently used for artemisinin analysis are not efficient enough. The conventional sample preparation methods involve at least two organic solvents [19] and at least two extraction steps [22,28,34], which make solvent recycling difficult and time-consuming, and introduce extra sources of variation. The microwave-assisted reflux extraction (MAE) [20] and pressurized solvent extraction (PSE) [18] can reduce extraction time considerably, but require special equipment. Supercritical fluid extraction (SFE) offers mild extraction conditions and eliminates further purification steps [18], but the instrument is costly. Nieuwerburgh et al. [29] established a rapid sample preparation approach, but the method was only tested in fresh leaves, and the results may vary due to the content of water in leaves.

The electron-capture detection (ECD) is a highly sensitive and selective method capable of detecting picogram amounts of the target analyte. Although there are some disadvantages of using ECD, such as the need of periodic check-up due to the millicurie amounts of radioactivity sealed in the detector, and the fact that the detector repair, disposal and replacement require a qualified technician, but this system is still commonly used and affordable, and has been primarily used to quantify organic molecules that contain electronegative functional groups. Mount and his collaborators [36] have developed a method to quantify artemisinin in whole blood by supercritical fluid chromatography (SFC) coupled to ECD, but the SFC is expensive and not widely used.

This work presented a sensitive and affordable method for determination of artemisinin in *A. annua* by GC-ECD and a simple sample preparation technique, here named 'single-solvent, one-step extraction'. This analytical procedure can be directly applied to analyze artemisinin from different plant parts, and can be adapted to quantify artemisinin from pharmaceutical drugs, commercial plant extracts, and even from individual leaves.

## 2. Experimental

### 2.1. Plant material and reagents

*A. annua* plants, included three different cultivars, were obtained from an experiment field of the Hunan Agricultural University (Changsha, Hunan, China). Artemisinin standard (98%, established by quantitative NMR, according to provider) was purchased from National Institute for Control of Pharmaceutical and Biologic Products (Beijing, China). *n*-Hexane was purchased from Changsha Chemical Reagent Company (Changsha, Hunan, China).

Artemisinin stock solutions were prepared by dissolving 25 mg artemisinin in 25 mL *n*-hexane and stored at  $-20^{\circ}\text{C}$ . The standard working solutions used to generate the calibration curve were prepared by serial dilutions of a stock solution with *n*-hexane.

### 2.2. Sample preparation

Leaves and flowers were collected from *A. annua* in September and October, respectively, and were frozen in liquid nitrogen for 30 s, dried in a freeze-drier (ALPHR1-2LD, Martin Christ Gefriertrocknungsanlagen, Germany) for 24 h, then passed through a stainless USA standard test sieve No. 14 mesh (1.4 mm openings), and stored in stoppered glass jars at  $4^{\circ}\text{C}$ , in the dark, before artemisinin analysis. Artemisinin extraction was performed by

immersing 0.1 g of sieved dry leaves to 10 mL of *n*-hexane, resulting in a plant material to solvent ratio of 1:100 to prevent solvent saturation. Three different extraction temperatures, ranging from 60 to  $69^{\circ}\text{C}$ , with three replicates, and five extraction times, with three replicates, ranging from 10 to 120 min were tested. The extract solution was cooled to room temperature, then was filtered through 0.2  $\mu\text{m}$  size nylon Millex-GN filters (Millipore, Bedford, MA, USA), pre-wetted with *n*-hexane, and attached to disposable 3-mL syringes. Filtered aliquots from the samples were transferred to GC flasks and were analyzed in the same day.

### 2.3. GC conditions

Analysis of artemisinin was performed by GC using an Agilent (Santa Clara, CA, USA) GC-6890N system with a micro-ECD system, a FID system, an autosampler and an Agilent data collection system (Rev. A. 09.01). Nitrogen was the carrier gas with a column flow rate of 2 mL/min, a split ratio of 3:1. The column was a HP-5 crossbond 95% dimethylpolysiloxane (Agilent), (30 m  $\times$  0.32 mm I.D., 0.25  $\mu\text{m}$  film thickness). Injector temperature was set at  $240^{\circ}\text{C}$ , and detector temperature set at  $300^{\circ}\text{C}$ . Oven temperature was programmed to start at  $180^{\circ}\text{C}$  (1 min), increasing  $0.8^{\circ}\text{C}/\text{min}$  to  $198^{\circ}\text{C}$ , then increasing  $30^{\circ}\text{C}/\text{min}$  to  $280^{\circ}\text{C}$ , then holding at that temperature for 10 min. The injection volume was set at 1.0  $\mu\text{L}$ . Possible problems, including malfunctioning autosampler or polluted liner, were monitored before and throughout the analysis by running an artemisinin standard of known concentration (normally 0.1 mg/mL) twice in the beginning, once every 10 samples, and at the end to check if changes in detector sensitivity and response would occur.

## 3. Results and discussion

### 3.1. Selection of GC detector

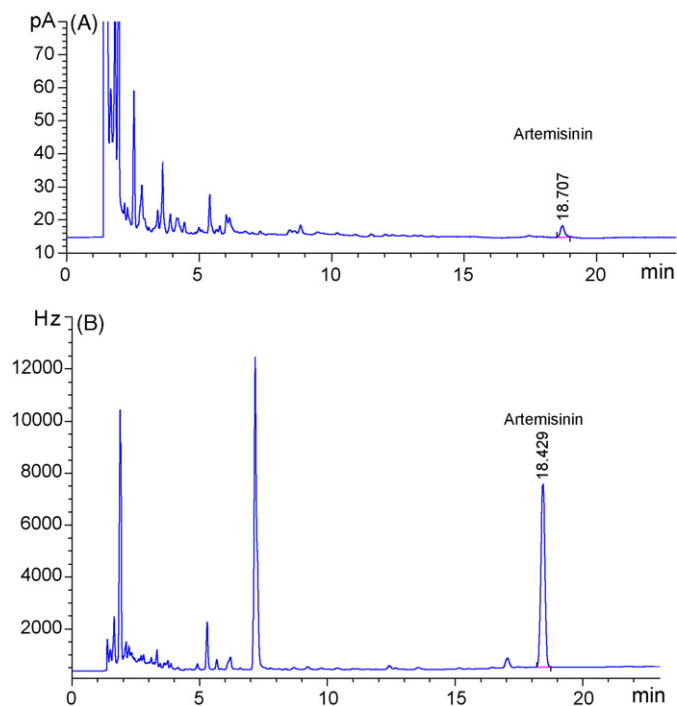
Before establishing the analytical procedure, the sensitivity of both FID and ECD to artemisinin was compared. We extracted the leaves of *A. annua* with *n*-hexane, and quantified artemisinin by GC-FID and GC-ECD, respectively, with one detector connected to the same column at a time, under the same GC condition. The identification of artemisinin was done through artemisinin standard. The chromatograms generated by GC-FID and ECD (Fig. 1A and B) produced a better S/N by ECD than by FID (ECD S/N = 3216 and FID S/N = 101, respectively) in response to a 1.0  $\mu\text{L}$  injection of a plant extract containing 57.3  $\mu\text{g}$  of artemisinin/mL. Consequently, ECD was chosen as the GC detection method for quantification of artemisinin.

### 3.2. Selection of the extraction solvent

Liquid extraction with toluene, *n*-hexane, chloroform, petroleum ether, or dichloromethane is currently the most common technique used for artemisinin extraction [18]. Because *n*-hexane is effective in extracting artemisinin, arteannuin B and artemisinic acid [19], it was selected as the extraction solvent for artemisinin. Artemisinin was directly analyzed by GC-ECD from the extraction solution without any additional separation or evaporating steps, as confirmed with artemisinin standards (Fig. 4) and leaf extracts (Fig. 1B).

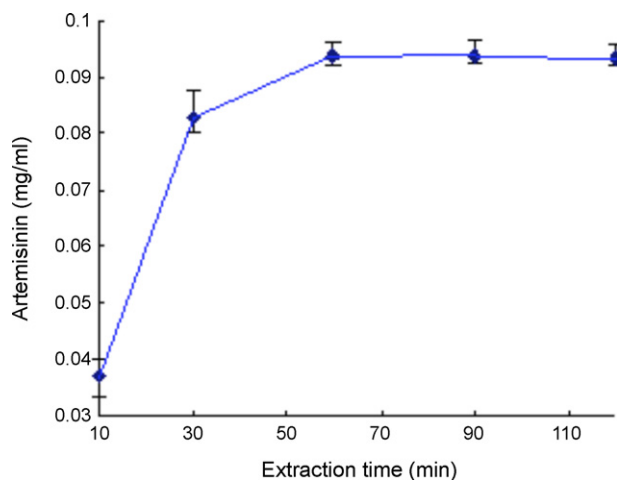
### 3.3. Effect of extraction time

With the extraction temperature set at  $69^{\circ}\text{C}$ , we investigated the effect of extraction time on extraction efficiency. Result (Fig. 2) showed that the concentration of artemisinin in the extraction solution increased significantly with prolonged extraction time up to 1 h, but not after that. To check whether there was a saturation

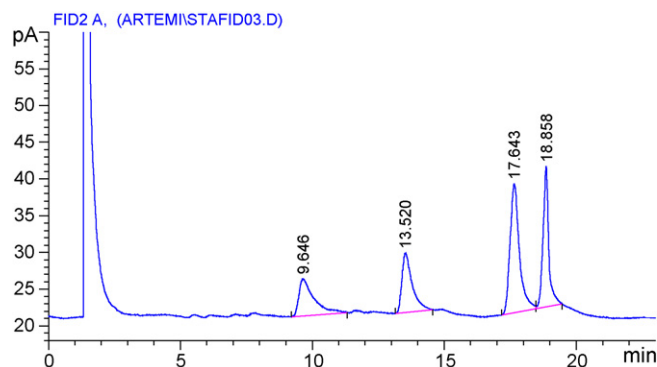


**Fig. 1.** (A) Chromatogram generated by GC-FID of a 1  $\mu$ L injection of an extract of *A. annua* leaves (10 mg dried leaves/mL of *n*-hexane) containing 57.3  $\mu$ g/mL artemisinin in *n*-hexane. Artemisinin peak area was 24.1 pA s. (B) Chromatogram generated by GC-ECD of a 1  $\mu$ L injection of an extract of *A. annua* leaves (10 mg dried leaves/mL of *n*-hexane) containing 57.3  $\mu$ g/mL artemisinin in *n*-hexane. Artemisinin peak area was 88,903 Hz s.

effect, two additional consecutive extractions of the same samples with fresh solvent were done. As a result (data not shown), the second extraction removed less than 3% of the artemisinin contained in sample and only trace artemisinin was detected from the third extraction. This indicated that over 95% of artemisinin have been recovered after the first 1 h of extraction and that the solute to solvent ratio of 1:100 prevented solvent saturation effects. These results demonstrated that one time extraction was enough for artemisinin, which agrees with previously published results obtained by refluxing with hexane [19], toluene [34] and petroleum ether [37]. Therefore, the optimal extraction time was set at 1 h.



**Fig. 2.** Effect of time on the extraction efficiency evaluated through the extraction of *A. annua* leaves with an artemisinin content of 0.94% (g/100 g dry weight). Plant material to solvent ratio was 1:100. Each extraction time was replicated 3 times with an average RSD of 3.1%.



**Fig. 3.** Chromatogram generated by GC-FID of a 1  $\mu$ L injection of artemisinin standard (1 mg/mL, artemisinin/*n*-hexane) under the same conditions used for the GC-ECD method. From left to right, the area of the four peaks ( $rt=9.6$ , 13.5, 17.6 and 18.8 min, respectively) were 122.1, 171.5, 400.9 and 300.2 pA s, respectively.

### 3.4. Effect of extraction temperature

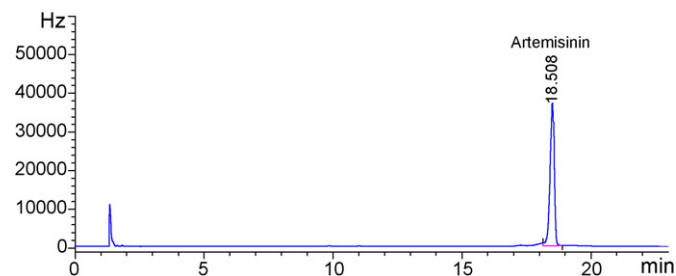
In order to obtain an optimal extraction condition, the relationship between extraction temperature and compound yield was investigated through the extraction of *A. annua* leaves with an artemisinin content of 0.94% (g/100 g dry weight). Each temperature point had three replications with an average relative standard deviation (RSD) of 3.9%. The results (data not shown) indicated that extraction with *n*-hexane at 60 and 65  $^{\circ}$ C allowed the extraction of more than 75% of the artemisinin from plant material, which may due to its sequestration solely in glandular trichomes [38,39]. However, the most effective extraction of artemisinin from plant material was performed at the solvent boiling point, 69  $^{\circ}$ C. This was confirmed by two additional extractions of samples of the same plant with similar results (data not shown).

### 3.5. Recovery

To assess the recovery rates of the sample preparation procedure, nine samples of 0.1 g dried leaf of *A. annua* were spiked with different volumes of a 10 mg/mL *n*-hexane stock solution of artemisinin. Immediately after evaporation of the hexane, all samples were refluxed with *n*-hexane at 69  $^{\circ}$ C for 1 h. Recovery rates of artemisinin ranged from 97 to 104% (Table 1). The established single-solvent one-step extraction sample preparation procedure allowed high artemisinin recovery, was suitable for rapid analysis of artemisinin in *A. annua* samples.

### 3.6. Thermal stability analysis

In order to investigate the thermal stability of artemisinin under the conditions used for GC-ECD, a series of standards, ranging from



**Fig. 4.** Chromatogram generated by GC-ECD of a 1  $\mu$ L injection of artemisinin standard (0.1 mg/mL, artemisinin/*n*-hexane).

**Table 1**

Recovery of artemisinin from samples spiked with increasing concentrations of an artemisinin stock solution

Samples (μg/mg)	Added amount (μg/mg)	Expected value (μg/mg)	Actual value (μg/mg)	Recovery (%)
4.12	2	6.12	6.07	99.1
5.58	2	7.58	7.38	97.3
6.22	2	8.22	8.01	97.4
6.83	5	11.83	11.92	100.8
7.16	5	12.16	11.99	98.6
7.39	5	12.39	12.58	101.5
7.75	10	17.75	18.48	104.1
8.27	10	18.27	17.82	97.5
9.13	10	19.13	19.77	103.4

**Table 2**Intra- and inter-day precision and accuracy ( $n=5$ )

	Actual concentration μg/mL	Detected concentration (mean ± SD) μg/mL	Precision RSD (%)	Accuracy (RE) (%)
Intra-day	30	28.8 ± 0.894	3.11	−4.05
	60	60.6 ± 1.178	1.94	+0.96
	100	100.9 ± 2.258	2.24	+0.87
Inter-day	30	28.8 ± 1.354	4.70	−3.99
	60	61.3 ± 1.567	2.55	+2.24
	100	99.6 ± 3.323	3.34	−0.42

RSD, relative standard deviation; RE, relative error.

0.5 to 1.5 mg/mL, were injected into a GC-FID system under the same condition as the ones used for the GC-ECD system. The results (Fig. 3) showed that, under the GC conditions, artemisinin was degraded into four major peaks detected by GC-FID, which is consistent with recently reported results [19]. Among the four peaks (Fig. 3), the fourth one matched the retention time ( $t_r=18$  min) of the single peak generated by ECD from artemisinin standards (Fig. 4). Although GC-MS was not available to us, the two peaks, which presented similar retention times by GC-FID and GC-ECD using the same column and oven conditions, were identified as the same compound. In GC-FID chromatogram, the fourth artemisinin peak represented 30% of the artemisinin peaks generated from a standard injection at a concentration of 1 mg/mL artemisinin, which was lower than the 55% calculated previously for the fourth artemisinin peak [19]. This difference may have been caused by the longer analysis time (Fig. 3) compared to that study [19]. There was a significantly high linear relationship ( $r^2=0.998$ ) between the area of the fourth peak generated by GC-FID and the concentration

of artemisinin. This indicated that the fourth peak (Fig. 3) could be used to quantify artemisinin, representing the true artemisinin content of samples, consistent with previous reports [19,31]. Because the single peak generated by GC-ECD (Fig. 4) corresponded to the fourth peak generated by GC-FID (Fig. 3), artemisinin quantification through this single GC-ECD peak represented the true content of artemisinin.

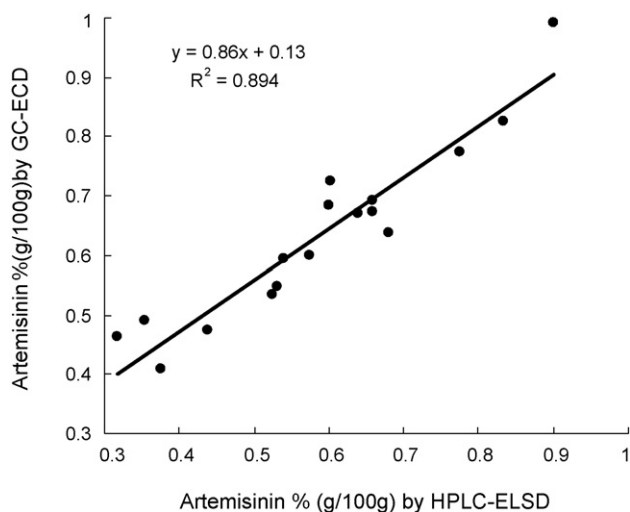
### 3.7. Method validation

#### 3.7.1. Calibration

A calibration curve for artemisinin was generated using artemisinin standards at different concentrations (0.02, 0.04, 0.06, 0.08, 0.1, 0.2 and 0.5 mg/mL) injected in the GC-ECD. The calibration curve of the standard artemisinin was obtained by plotting the artemisinin concentration (mg/mL) on the X axis against the peak area (Hz s) on the Y axis. Linear regression resulted in the following equation:  $Y=18.28X-0.16$  with  $r^2=0.9890$ , indicating a significant linear relationship from 0.02 to 0.5 mg/mL. Results further demonstrated the single peak generated by GC-ECD (Fig. 4) could be used to quantify artemisinin.

#### 3.7.2. Precision and accuracy

The precision was estimated by determining repeatability and intermediate precision. Regarding repeatability, 1 μL of 0.5 mg/mL standard of artemisinin was injected five times consecutively by an autosampler. The RSD of retention time was less than 0.7 and 4.1% for peak area. The intermediate precision, which is also called intra- and inter-day precision, was determined by analyzing five replicates of three quality control samples at concentrations of 30, 60 and 100 μg/mL. The quality control samples were prepared by standard solutions as a single batch on the same day at each concentration, and then divided into aliquots that were stored at  $-20^\circ\text{C}$  until analysis. For intra-day assay precision, five replicates of quality control samples at each concentration were assayed all in the same day. The inter-day assay precision was determined by analyzing the quality control samples on five different days. Five replicates at each concentration were assayed per day. The precision for measurement of artemisinin was summarized in Table 2. The RSD of artemisinin ranged from 1.9 to 3.1% for intra-day and 2.6 to 4.7% for inter-day, respectively. The accuracy was evaluated by direct comparison to the reference standard. The relative error (RE) of



**Fig. 5.** Correlation of artemisinin content (w/w%) of 17 plant materials analyzed by both GC-ECD and HPLC-ELSD. These plant materials consisted of leaves from different parts of 3 cultivars of *A. annua*. Their artemisinin content ranged from 0.317% to close to 0.901% (w/w).



artemisinin ranged from –4.1 to 1.0% for intra-day and –4.0 to 2.2% for inter-day, respectively (Table 2).

### 3.7.3. Comparison with HPLC-ELSD

To further validate the proposed GC-ECD method, the well-characterized HPLC-ELSD method [19,20] was chosen. Seventeen plant samples, consisting of leaves from different parts of three cultivars of *A. annua*, ranging from 0.3% to close to 1%, were extracted and analyzed for their artemisinin content by both GC-ECD and HPLC-ELSD. The HPLC-ELSD analysis was performed by a Shimadzu LC-10ATVP (Kyoto, Japan) with an Alltech ELSD-2000 (Deerfield, IL, USA), following the reported procedure and chromatography condition [19]. Artemisinin content was calculated according to the calibration curve generated by the known concentration of artemisinin standards through their peak area. A significant correlation ( $r^2 = 0.894$ ,  $p < 0.001^{**}$ ) was obtained when the concentration (g/100g) of artemisinin from the 17 plant samples was compared by GC-ECD and HPLC-ELSD (Fig. 5). The results obtained by GC-ECD agreed with the ones obtained by HPLC-ELSD.

### 3.7.4. Limit of quantification (LOQ) and limit of detection (LOD)

LOQ was defined as the lowest concentration of artemisinin at which the signal-to-noise ratio (S/N) was larger than 10 ( $S/N \geq 10$ ), and LOD was defined as  $S/N \leq 3$ . The measured LOQ and LOD values of the described method were 9 and 3  $\mu\text{g/mL}$ , respectively, which, on a ng/column basis, was over 9 times more sensitive than the LOD of artemisinin by GC-FID, and over 15 times more sensitive than the LOD for artemisinin by HPLC-ELSD recently reported [19].

## 4. Conclusions

Under the GC conditions used for this method, artemisinin was degraded to four breakdown products (peaks), while only one peak, identified as the fourth peak in GC-FID, occurred in GC-ECD. A linear relationship was observed between artemisinin concentration and the area of the fourth peak. Artemisinin was measured by GC-ECD on the basis of this peak represented the true artemisinin content of the samples. A significant correlation was found between the results obtained by GC-ECD and HPLC-ELSD. This study showed that GC-ECD was sensitive and affordable for quantification of artemisinin, although artemisinin was not analyzed as a whole molecule due to its thermal instability. In this paper, we also developed an efficient analytical procedure for determination of artemisinin in *A. annua* by GC-ECD. The 'single-solvent, one-step extraction' sample preparation technique proposed presented recoveries of 97% or higher since it eliminated additional steps (such as solid phase extraction, centrifugation, evaporation, and so on) which are time-consuming, and constitute sources of recovery losses. In addition, this one-solvent extraction makes solvent recycling easier, which will reduce organic solvent waste disposal. This method requires only 100 mg of plant material, and can be easily applied to determine the content of artemisinin in an individual leaf, which is important to artemisinin biosynthesis studies [35]. The developed analytical procedures may be adapted to quality control methods of artemisinin in pharmaceutical drugs and commercial plant extracts.

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